6333

FINAL REPORT ON

CONTRACT NO DA-92-557-FEC-34525

INCLUSIVE DATES 15 January 1962 TO 14 January 1963

SUBJECT OF INVESTIGATION

STUDIES ON
DEOXYRIBONUCLEIC ACID
METABOLISM

RESPONSIBLE INVESTIGATOR

Dr. Yasuyuki Takagi

Professor of Biochemistry
Kanazawa University Medical School
Kanazawa, Japan

U.S. Army Research & Development Group (9852) (Far East)
Office of the Chief of Research and Development
United States Army
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AD Div 16/3

Kanazawa University Medical School (Japan) STUDIES ON DEOXYRIBONUCLEIC ACID METABOLISM by Yasuyuki Takagi. Final Report No. 2, 15 Jan 62 to 14 Jan 63. 12 p. illus. tables 6 refs. (Contract DA92-557-FEC-34525) Unclassified report

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Mitomycin C exerts inhibitory action on hereafter DNA formation of the cells, even when exposed to the antibiotic during the period that the DNA replication is completely blocked by the lack of a precursor. Thus it was suggested that the regulatory mechanism to initiate the DNA synthesis is actually operating in the cells and may be susceptible to the action of Mitomycin C.

One possibility of such mechanism of DNA polymerization is the state of complex of DNA and DNA-polymerase in the cells, since it was suggested that DNA-synthesizing activity is associated with DNA in the cells.

Other experiments performed in relation to the biochemical mechanism of mutation demonstrated that thymine marker of Hfr H and C locates on the chromosome between Sm and H markers. (Author)

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D-I-S-T-R-I-B-U-T-I-O-N

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The Abstract of the Final Report No.2

The purpose of this investigation is to clarify the real biochemical mechanism of DNA formation involved in cell division on the basis of enzyme-chemical studies on DNA polymerase. Main efforts were made to analyze the mode of action of mitomycin C on DNA polymerization reaction in Escherichia coli, since this compound exhibits the primary inhibitory action on this step in the DNA formation system. However the experiments with cell-free extract indicated that the polymerase itself is not interferred by the presence of the antibiotic, nor MC causes any damage or alternation in the DNA molecule in the cells resulting in the loss of its activity as primer in polymerization reaction. While MC exerts inhibitory action on hereafter DNA formation of the cells, even when exposed to the antibiotic during the period that the DNA replication is completely blocked by the lack of a precursor. Thus it was suggested that the regulatory mechanism to initiate the DNA synthesis is actually operating in the cells and may be susceptible to the action of MC.

One possibility of such mechanism of DNA polymerization is the state of complex of DNA and DNA-polymerase in the cells, since it was suggested that DNA-synthesizing activity is associated with DNA in the cells.

Another experiments performed in relation to the biochemical mechanism of mutation demonstrated that thymine marker of Hfr H and C locates on the chromosome between Sm and H markers.

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CONTENT

THE FINAL REPORT NO. 2

1. A STATEMENT OF THE PROBLEM

In the past decade evidences obtained in various fields of biology have witnessed the universal acceptance of the idea that deoxyribonucleic acid (DNA) is the substance in the chromosomes of the cell nucleus which governs the heredity of the cell. Thus it is self-explaining that a knowledge of DNA biosynthesis is essential in order to understand the chemical events in the cell division, or to formulate the chemical basis for most genetic phenomena. One attack to this problem has been nutritional and genetic studies with intact cells, and many interesting experiments along these lines have been performed with animal tissue and bacteria, but these studies have not defined any mechanism and sometimes caused more serious confusion. Another approach has been the isolation from cells of separate enzymes, each of which effects a single, chemically rational step leading to the total formation of the DNA molecule. It is well known that Kornberg and his colleagues have reported the elegant studies on the enzymatic synthesis of the DNA molecule. Nevertheless, it has been very obvious that there is no bridge between these enzymatic studies and the observations on DNA replication in intact cells, and our knowledge on the real biological events in DNA formation during cell division is very scanty. For instance, we do not understand what will initiate DNA synthesis in intact cells. Furthermore we do not know the real significance of Kornberg's enzyme in the DNA formation system of the intact cells, since enzymatic synthesis of biologically active DNA molecule using this enzyme has not been succeeded. Therefore this research was undertaken to reduce the distance between the data derived from the analysis of intact cells and the purified enzymes in an attempt to clarify the real mechanism of DNA formation in the cells.

2. ANALYSIS OF THE PROBLEM

It has been well established that DNA is not synthesized continuously and that the total amount of DNA of the chromosomes increases to a level twice as high as that of a normal nucleus only during the process of cell division. That is, cell division must be preceeded or accompanied by a synthesis of DNA, since the two daughter cells formed have the same amount of DNA in their nuclei as the mother cell. Though the exact moment of this synthesis is still a matter of argument, the conception of the formation of DNA in late inter-

phase seems to be accepted by most authors at the present time. Thus it is expected that the study on the process priming the DNA synthesis may give a clew at the same time to solve the complicated mechanism inducing the cell division. Accordingly in this research project one of the most intriguing aspect of DNA formation, the mechanism initiating this synthesis in the cell will be most sharply focused in relation to the biochemical reactions involved in cell division.

One of the trials in this direction is to analyze effects of irradiation or treatment with various chemicals such as N-mustard which results in interference of DNA synthesis as well as the induction of mutations. During the first contract period, efforts were made to localize the site of attack on the DNA synthesizing system by mitomycin C (MC), since this antibiotic was recently indicated that the nuclear apparatus of the cells may be particularly susceptible to the action of this compound, because of its inhibitory action on DNA formation and of its mutagenesis, the properties similar to ultraviolet effect. Our experimental data presented evidences that the DNA polymerizing step (DNA polymerase) in intact cells may be the primary site of the action of the antibiotic. However another experiment with a cell-free extract prepared by the method of Kornberg indicated that the incorporation of tritiated thymidine into DNA molecule is not supressed in the presence of MC at the low level used for the studies with intact cells. These results suggest strongly that the antibiotic does not interfer the polymerase itself but some mechanism which operates to spark the synthesizing enzyme system in intact cells in order to initiate DNA synthesis may be particularly sensitive to the action of MC. Hence during this contract period, the effect of MC on the DNA synthesizing system was analyzed on the basis of informations obtained in enzymatic studies in order to reveal the initiating system of DNA formation in intact cells and to confirm the significance of Kornberg's polymerase during the process of cell division.

Another problem in relation to the mechanism of the biosynthesis of DNA is to find out what type of biochemical change will lead to mutation. In spite of the great number of investigations and the large amount of thought that has been applied to genetic mutation we still do not really understand what type of change leads to mutation. We can not tell whether changes in particular parts of the DNA molecule will lead to mutation although it is believed that first step of mutation is a failure of DNA copying. Therefore events leading to genetic mutation will have to be studied from the biochemical facts of DNA metabolism.

3. OUTLINE OF EXPERIMENTAL PROCEDURE

a. Effect of Mitomycin C on the Synthesis of DNA.

(1) Effect of Mitomycin C on the Primer DNA in the Synthesis of DNA. One possibility concerning mechanism to spark DNA synthesis is the state of primer which was shown with the purified Kornberg's enzyme to be indispensable for the occurance of the polymerizing reaction and to be a template for newly synthesized DNA molecule. Therefore effects of MC on the priming activity of DNA were investigated at the enzyme level. In our previous experiments on the incorporation of labelled thymidine into DNA with a cell-free extract, DNA prepared from other source (not exposed to the antibiotic) was added to the incubation mixture as the DNA primer. Thus it is presumed that such normal DNA preparation may be capable to act as the primer even in the presence of the antibiotic, while in the experiments with intact cells DNA in the cells may have lost the ability to exert a priming action due to the damage or somewhat alternation in its structure caused during the prior replication process of DNA by the exposure to the antibiotic. Accordingly, DNA preparations were obtained from Escherichia coli or Alcaligenes fecalis which ceased DNA formation by the previous exposure to the antibiotic, and their priming activity was comapred with those of DNA extracted from the unexposed control bacteria.

(2) Isolation of Tritiated Mitomycin C. The detailed studies concerning the fate of MC when exposed to the cells may be of great aid in understanding the mechanisms of action of the antibiotic. In particular it is absolutely requested to reveal whether MC is permeated into the bacterial cells and to investigate if the compound is bound to DNA molecule of the cell in order to exhibit the inhibitory action on DNA synthesis. Thus efforts were made to label MC with tritium for the purpose of carrying out this kind of experiment.

(3) Effect of Mitomycin C on Escherichia coli 15 T⁻ under Thymine-starvation. Maalφe demonstrated recently some evidence supporting the assumption that protein and RNA synthesis is requested prior to a replication cycle of DNA and that the DNA formation is completed during the process of replication, once initiated, even in the absence of concomitant synthesis of cytoplasmic materials,

then stops because of a lack of reactions necessary to spark a next replication cycle of DNA. Therefore the factors initiating DNA formation should be searched from the studies on the nature of protein or RNA prerquisite to DNA synthesis. Hence it was tried during the contract period to confirm whether MC primarily interrupts the process of formationof such protein or RNA. The experiments were conducted with thymine-requiring mutant strains of Escherichia coli 15, and the effect of MC on the bacteria under thymine-starvation was compared with that in the complete medium supplemented with thymine, since it was expected that DNA synthesis in the cells may be separated from the process of other biological specific synthesis under the condition of thymine-starvation.

b. Fractionation of DNA synthesizing System from Bacterial Extract. One trial to search the regulatory mechanism of DNA formation may be to obtain simple fraction synthesizing DNA, in which possible effects from many other unknown factors can be excluded, and to analyze the intracellular distribution of DNA synthesizing system, DNA or DNA-protein complex, and DNase. Then the interrelation existing within the triad in the cells, the factors initiating DNA formation, relation between DNA formation and the energy metabolism supporting it, and the alternative pathway systems operating in the DNA formation process will be clarified from analysis of the results obtained with such subcellular or more disrupted fraction prepared from cells under various physiological conditions. Accordinglyefforts were made to prepare such fraction containing DNA synthesizing system from cell-free crude extract by differential centrifugation or column chromatography.

c. The Chromosomal Site of the Thymine Locus. During the first contract year a new method to select thymine requiring mutants of various strains such as Escherichia coli, Salmonella typhimurium was established by inoculating the organisms in glucose Simmons' medium supplemented with aminopterine and thymine. Since this method made possible to separate many thymine requiring strains, the genetical nature of the mutant-whether chromosomal or cytoplasmic- was analyzed by means of the technique of bacterial recombination.

4. RESULTS OBTAINED

a. Effect of Mitomycin C on the Synthesis of DNA.

(1) Effect of Mitomycin C on the Primer DNA in the Synthesis of DNA. Bacteria harvested during the

logarithmic growth phase were treated with various amount of MC for different time intervals, then DNA was extracted and purified using several kinds of procedure described below.

(a) The firstprocedure was adapted from the method of Marmur. Cells were disrupted by sodium lauryl sulfate, and deproteinized repeatedly by shaking vigorously with chloroform-isoamylalcohol mixture. The contaminating RNA was then removed by the action of RNase and DNA was selectively precipitated with isopropanol. The DNA thus obtained was almost free of protein and RNA.

(b) Protein in the culture was removed from the sodium lauryl sulfate lysate described above by means of papain digestion. Usually papain was added to lysate in order to make a final concentration of 1 per cent and after incubation of 4 hours at 37° the digest was dialyzed overnight against diluted phosphate buffer. These procedures were repeated three times, following which RNA was removed by RNase treatment.

(c) Cells were lysed by repeated freezing and thawing, then deproteinized with phenol.

(d) Nucleic acids were extracted twice from the cells with sodium chloride, then purified by repeating the precipitation with alcohol. The preparation obtained was the most crude one.

These DNA preparations were heated for 10 minutes at 90° according to the description of Bollum, who demonstrated that native DNA preparations are inactive as primers in the polymerization by purified calf thymus polymerase and become primers after heat-denaturation. The assay of priming activity of DNA preparations was performed by the procedures of Bollum. Incubation mixture consisted of cell-free crude extract of calf thymus. DNA preparation obtained above, tritiated thymidine, monophosphates of deoxyadenosine, deoxyguanosine and deoxycytidine, magnesium ion, 3-phosphoglycerate, and adenosine triphosphate. The formation of the DNA molecule was determined by the incorporation of tritium into acid-insoluble fraction. Therefore the DNA synthesizing system includes the operation of at least two separate kinds of enzyme: kinases responsible for phosphorylation of deoxyribonucleoside monophosphates to

triphosphates, the direct substrates of the enzymatic formation of DNA molecule, and DNA polymerase.

As found in Table I heat denatured bacterial DNA exhibited activity as the primer of the polymerizing reaction though much lower than the denatured salmon sperm DNA used as a control. In some case the synthesis of DNA with the DNA preparation of MC-treated cells added as the primer occured at about the half level of that with DNA from the untreated organisms, but the rate of inhibition of priming activity by MC was not in complete proportion to the time period of exposure to the antibiotic. Thus in spite of various trials, it was found that the priming activity of the DNA preparations extracted from MC-treated cells was at the almost same level, compared to that from the control untreated organisms.

(2) Isolation of Tritiated Mitomycin C. MC was exposed to tritiated hydrogen gas for several weeks and the labelled compound was purified by extraction with organic solvent and finally by paper chromatography. However no antibiotic activity was foundin the tritiated compound thus obtained.

(3) Effect of Mitomycin C on Escherichia coli 15 T^- under Thymine-starvation. Escherichia coli 15 T^- harvested during the logarithmic growth phase was preincubated in the absence of thymine for various time period indicated in the Table II, in order to starve thymine. Cells were then devided into four portions: A was immediately subjected to counting the number of colony former and determination of DNA content; B was suspended in a medium lacking thymine; C was exposed to a medium containing MC at a level of 1 μg per ml. and lacking thymine; D was suspended in a medium containing 1 μg per ml. of MC and 2 μg per ml. of thymine. B, C, and D portions were incubated for 7 minutes, centrifuged, and transferred to a fresh medium supplemented with thymine. After 45 minutes of reincubation the number of colony former and DNA content in each portion was determined. As found in A portions of Table II, number of colony former was progressively decreased with increasing length of preincubation time for thymine-starvation, presenting the thymineless-death, and the numbers were not elevated by reincubation in the medium supplemented with thymine (B portions). A marked reduction of the viable count

was observed after exposure to MC during incubation for 7 minutes following thymine starvation, at the same level regardless of the presence or absence of thymine in the medium (C and D portions). In addition the relative amount of DNA in the culture is shown in the Table, in which the content of DNA immediately after preincubation for thymine-starvation (A portions) is represented by 100. The amount of DNA reached to a level more than twice by reincubating the thymine-starved cells in the complete medium supplemented with thymine (B portions), while the rise of DNA content was depressed considerably when the cells were exposed to MC prior to reincubation, also at the same level irrespective of the presence or absence of thymine.

b. Fractionation of DNA Synthesizing System from Bacterial Extract. Escherichia coli was harvested during the logarithmic growth phase and washed once with cold distilled water. The packed cells were ground with quartz sand and the resulting paste was extracted with tris-Mg buffer (pH 7.4). The insoluble residue was removed by centrifugation at 30, 000 x g for 30 minutes. The extract was then subjected to a 105, 000 x g centrifugation for 2 hours in order to remove ribosome fraction, and the supernatant was again centrifuged at 105, 000 x g for 4 hours. The supernatant obtained was devided into 3 portions from top to botton (referred to as S_1, S_2, S_3). The pellet was resuspended in tris-Mg buffer (referred to as B). Each fraction was analyzed for DNA synthesizing activity and DNA content. For the assay of DNA polymerizing activity the fraction was incubated with triphosphates of deoxyadenosine, deoxyguanosine and deoxycytidine, tritiated thymidine triphosphate in the presence of Mg^{++}. The formation of DNA molecule during incubation was determined by counting tritium incorporated into acid-insoluble fraction in liquid scintillation spectrometer. DNA in the fractions was measured by the method of Burton. As shown in Fig 1, most of the polymerizing activity was found in pellet fraction (B) and in the bottom layer of supernatant (S_2). None was detected in the top layer (S_1). The distribution of DNA indicated a same pattern as that of polymerizing activity. In the experiments repeated, polymerizing activity was assayed using the incubation medium supplemented with salmon sperm DNA added as the primer, since it was suspected that no activity observed with the S_1 fraction may be due to the lack of DNA in the fraction indispensable for the enzymatic synthesis as the primer. However no formation of DNA was again demonstrated with the top layer of the supernatant (S_1) under the conditions. (Fig. 2) Pellet fraction from cell extract prepared in sonic

oscillator has always far less activity compared to pellet obtained as above by grinding cells with quartz sand (Fig. 3). These findings seemed to suggest that DNA polymerizing system might be associated with DNA in some way in the cells, and prompted us to search for DNA-polymerase complex. In the next experiments, the 105, 000 x g supernatant of cell free extract prepared by grinding Escherichia coli with quartz sand was put on Sephadex G-200 column (1 x 60 cm) and eluted with the same tris-Mg buffer. Since DNA polymerizing system was found to sediment faster than other cellular protein, this dextran gel known to separate substances with molecular weight of lower than 200, 000 was employed in order to purify the system from other cellular constituents. The rate of flow of the eluent was 1-2 ml per hour. The eluate was collected in 2 ml portion via an automatic fraction collector with a drop-counting mechanism and subjected to read UV absorption and to assay polymerizing activity. The results were indicated in Fig. 4. As predicted, the polymerizing activity was found only in the first peak of protein eluted from the column. The formation of DNA was not demonstrated with a broad second peak which mainly consited of soluble protein. In addition it was observed that DNA was eluted in completely same fractions as the polymerizing activity was.

c. The Chromosomal Site of the Thymine Locus. Experiments were performed in two parts on the genetic transfer of thymine requiring character.

(1) Genetic Analysis of Recombinants. Hfr C and F^- W1177 thy_2 H^- (TLB_1^- Lac^- Sm^r) were grown, as donor and recipient strain respectively, to 4.2-4.3 x 10^8 cells per ml in GS-PYT medium (glucose-Simmons' medium supplemented with polypepton 8 gm , yeast extract 1 gm, thymine 20 mg per liter) at 37° C. The cultures were then centrifuged, resuspended in fresh medium. 0.1 ml of donor strain suspension was mixed with 0.9 ml of recipient strain suspension and incubated at 37° C for 3 hours. The mixture was then washed twice, diluted and plated on the agar media containing the nutrients required by both parents except methionine and histidine. 266 colonies of M^+ H^+ recombinant appearing were picked up and unselected characters such as maltose fermentation ability, mannitol fermentation ability, streptomycin sensitivity and thymine requirement of the recombinants were assayed. The number of recombinant with various characters was illustrated in Table III. The results may be explained consistently, when assumed that thymine marker is located between Sm and H markers on the chromosome.

(2) The Kinetics of Formation of Recombinants. Donor and recipient strains grown to 2-5 x 10^8 cells per ml in GS-PYT medium were centrifuged, resuspended in fresh medium, and mixed in order to give ratio of 9 : 1. At various time intervals after mixing bacterial conjugation in the mixture was blocked by shearing in syringe needle, and plated on the selective media. Selection was made by the concerning marker and streptomycin resistancy. The plates were incubated for about 40 hours at 37° C and the recombinant colonies appearing were counted. The experiments with Hfr H (M^-), and Hfr C (M^-) as donor strain and F^- W1177 thy^- H^- as recipient strain were indicated in Figs.5 and 6. Hfr H and C transfers the markers in the following order: T, L, Az, T_1, ·· and Lac, Pro, T_1, Az, ····, respectively. Therefore the results support again the assumption that thy marker locates between Sm and H markers, if thy marker lies on the chromosome of Escherichia coli. During the experiments one Escherichia coli 58-161 which transfers thymine marker with remarkably high frequency was isolated (Figs. 7 and 8). The characterization of this strain called 58-161 Hfthy is now in progress.

5. CONCLUSION DRAWN

a. Thespecific inhibitory action of MC on DNA formation may be not due to the damage or somewhat alternation in the DNA molecule caused by exposure to the antibiotic resulting in the loss of priming action in polymerizing reaction, as far as analyzed in the experimental system of DNA formation using cell-free extract of calf thymus as enzyme preparation.

b. MC loses its antibiotic activity during contact with hydrogen gas, probably because of reduction or some change of its molecule.

c. MC exerts hereafter inhibitory action on colony forming ability and DNA synthesis of Escherichia coli 15 thymine-requiring strain, even when bacteria were exposed to the antibiotic while the DNA replication is blocked under the condition of thymine-starvation.

d. The distribution of DNA synthesizing system coincides with the distribution of DNA during fractionation by differential centrifugation or column chromatography, supporting the assumption that DNA polymerase may be associated with DNA in some way in intact cells.

e. Thymine marker of Hfr H and C locates on the chromosome between Sm and H markers. However one strain of 58-161 transfers the marker with exceptionary high frequency, suggesting different kind of genetic behavior.

6. CONTRIBUTIONS TO THEORY

Since Kornberg reported the enzymatic formation of DNA, there are many lines of experimental evidence supporting that the DNA polymerase is actually operating for the synthesis of DNA in intact cells. However the regulatory mechanism of the polymerase in the cells in order to initiate DNA production only prior to the cell division is presently unknown. In our previous studies it was demonstrated that MC exhibits selective inhibitory action on DNA formation without affecting the formation of either RNA or protein in Escherichia coli. Hence mode of MC action was analyzed on the basis of enzyme chemical studies performed by Kornberg in order to clarify the real mechanisms of DNA formation involved in cell division. The experiments carried out during the first contract year suggested strongly that the DNA-polymerizing step is the primary site of the action of MC in intact cells. On the other hand, another experiment with cell-free extract indicated that the antibiotic has not inhibitory effect on polymerase itself. In addition the experimental data obtained during the second year of this contract showed that DNA preparations extracted from Escherichia coli, previously exposed to MC, exhibited the priming action in the polymerization at almost the same level with that obtained from the unexposed control bacteria. The results indicate that the inhibitory action of MC on DNA formation in intact cells may be not due to the damage or somewhat alternation in the DNA molecule indispensable in the polymerization reaction as the primer. One possibility is that this assay system may not be suitable to detect the real primer DNA molecule in the cells. That is, even the DNA molecules damaged or somewhat altered in its structure by the exposure to MC and lost their biological activity as primer DNA in intact cells may still be able to prime the DNA synthesis in the studies with cell-free extract, which follow simply the incorporation of thymidine into acid-insoluble fraction in the presence of other three kinds of deoxyribonucleoside monophosphate. However it was denyed, since intact cells lost completely the ability to incorporate thymidine into acid-insoluble fraction under the same conditions after exposure to the antibiotic. Other finding that the exposure of Escherichia coli to MC does not cause any suppression of inducible formation of β-galactosidase may strengthen the assumption of no damage or alternation in the DNA molecule in the cells by the antibiotic. Thus all efforts to reveal the site of the inhibitory action of MC on DNA synthesis at the enzyme

level were unsucceddful. The discrepany between the *in vitro* and *in vivo* studies concerning the effect of MC on DNA synthesis may be well explained by assuming the existence of the regulatory mechanism particularly susceptible to the action of the antibiotic. This possibility was further supported by the inhibitory action of MC on the later DNA synthesis of the cells exposed to the antibiotic when the DNA replication is blocked by the lack of a precursor, thymine.

The nature of the regulatory mechanism of DNA polymerase is obscure, although several evidences for the formation of protein and RNA prerequisite to DNA formation are known. Another possibility is the state of complex of DNA and DNA-polymerase, since it was suggested that DNA-synthesizing activity is associated with DNA in the cells. Thus the trials to analyze the regulatory mechanism of DNA synthesis in the cells on this line are in progress.

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APPENDIX "A"

TABLE I

Effect of Mitomycin C on the Primer DNA in the Synthesis of DNA

Experimental No.	Source of DNA	Method of Purification of DNA	Radioactivity in Acid-insoluble Fraction (counts/minute)
1	MC-treated *E. coli*	(a)	940
	Untreated *E. coli*	(a)	670
	Salmon Sperm		5,710
2	MC-treated *Alc. fecalis*	(b)	230
	Untreated *Alc. fecalis*	(b)	320
	Salmon Sperm		2,880
3	MC-treated *E. coli*	(b)	310
	Untreated *E. coli*	(b)	350
	Salmon Sperm		1,730
4	MC-treated *E. coli*	(c)	470
	Untreated *E. coli*	(c)	340

APPENDIX "B"

TABLE II

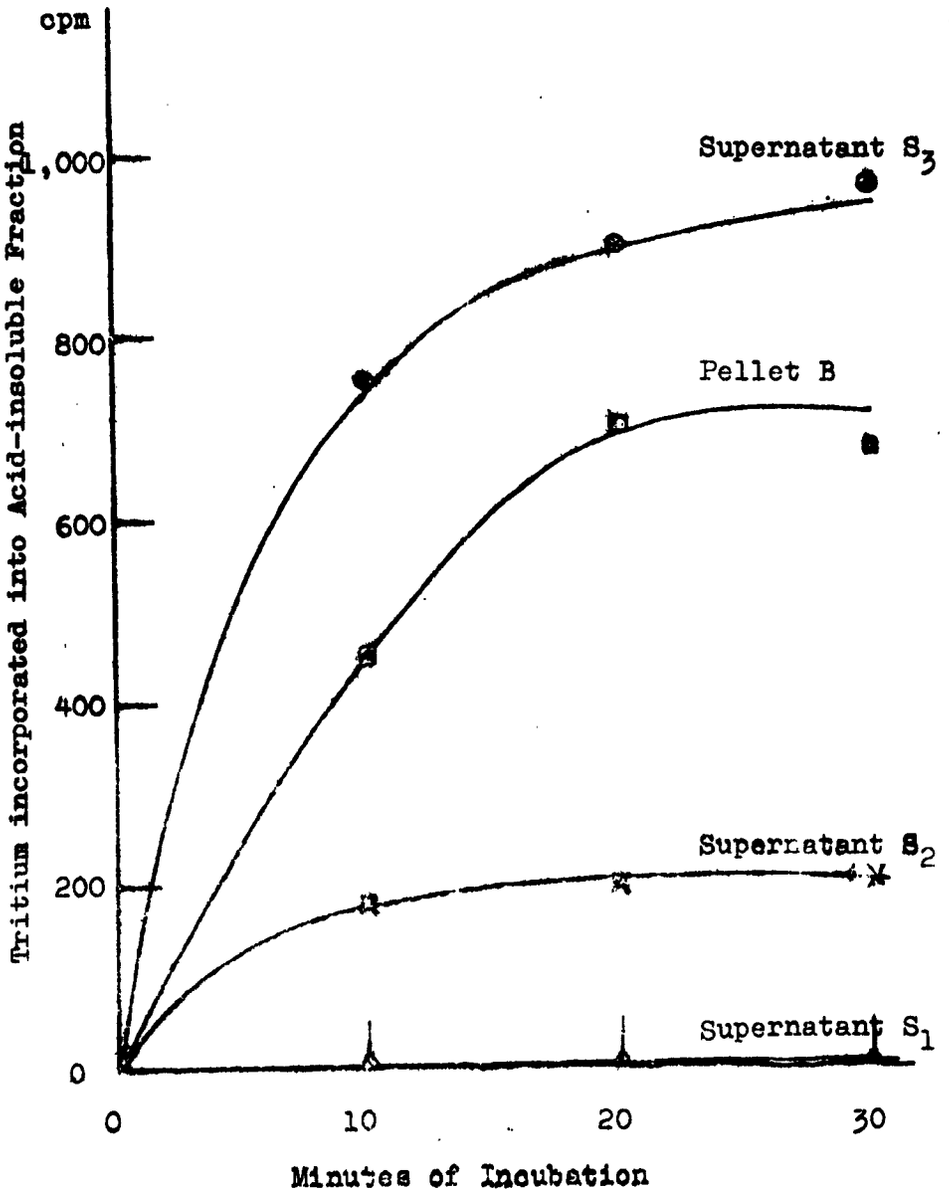
Effect of Mitomycin C on E. coli 15T⁻ under Thymine-starvation

Preincubation Time Thymine-starvation (min.)	Portion	Supplements to the Incubation Medium	Relative Amount of DNA	Number of Colony Former (per ml.)
0	A		100	5.0×10^8
	B	Thy - MC -	170	7.9×10^8
	C	Thy - MC +	140	8.3×10^6
	D	Thy + MC +	139	1.3×10^7
45	A		100	3.1×10^8
	B	Thy - MC -	268	3.4×10^8
	C	Thy - MC +	189	5.2×10^5
	D	Thy + MC +	217	5.2×10^5
90	A		100	2.2×10^7
	B	Thy - MC -	220	2.1×10^7
	C	Thy - MC +	138	1.7×10^5
	D	Thy + MC +	110	1.0×10^5

APPENDIX "C"

Fig. 1

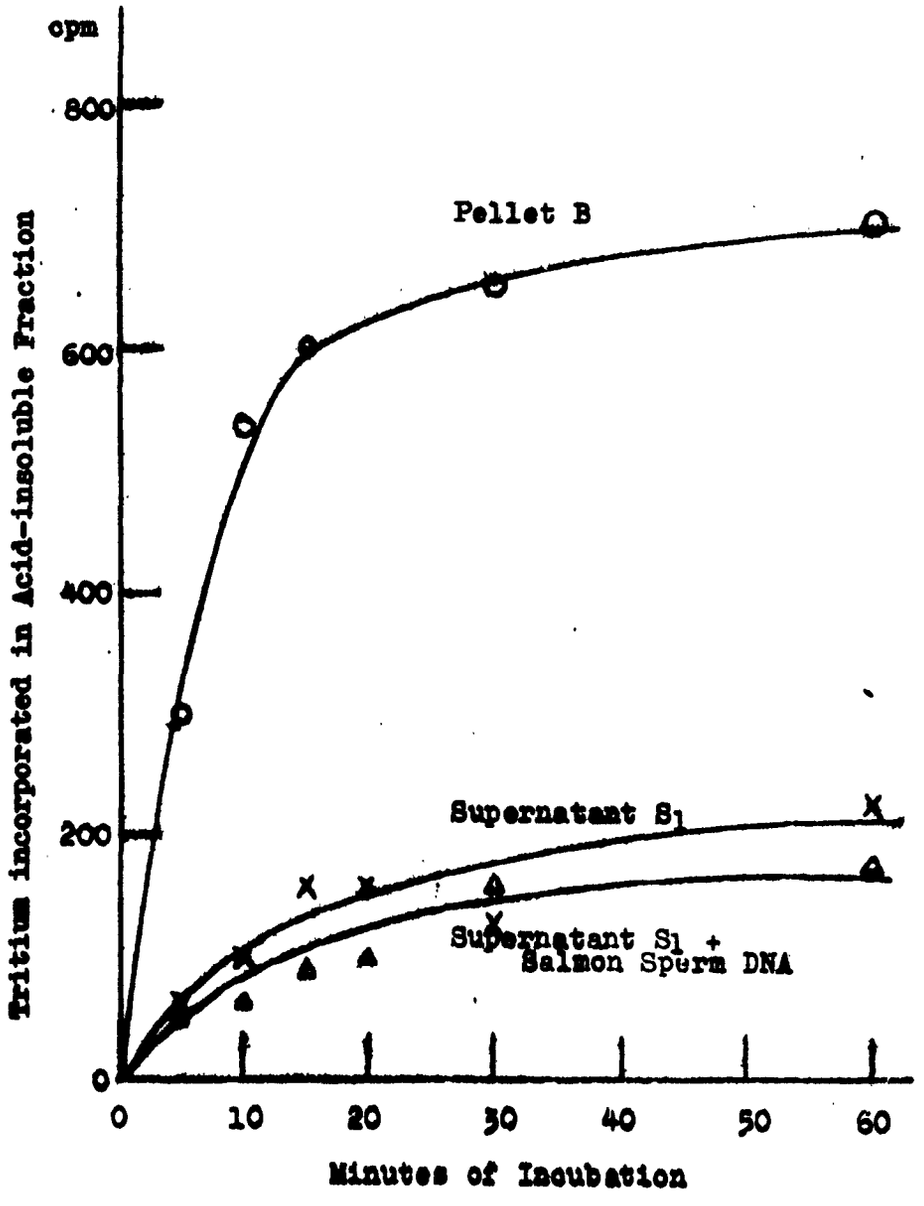
Distribution of Polymerizing Activity after Differential Centrifugation of Bacterial Extract



APPENDIX "D"

Fig. 2

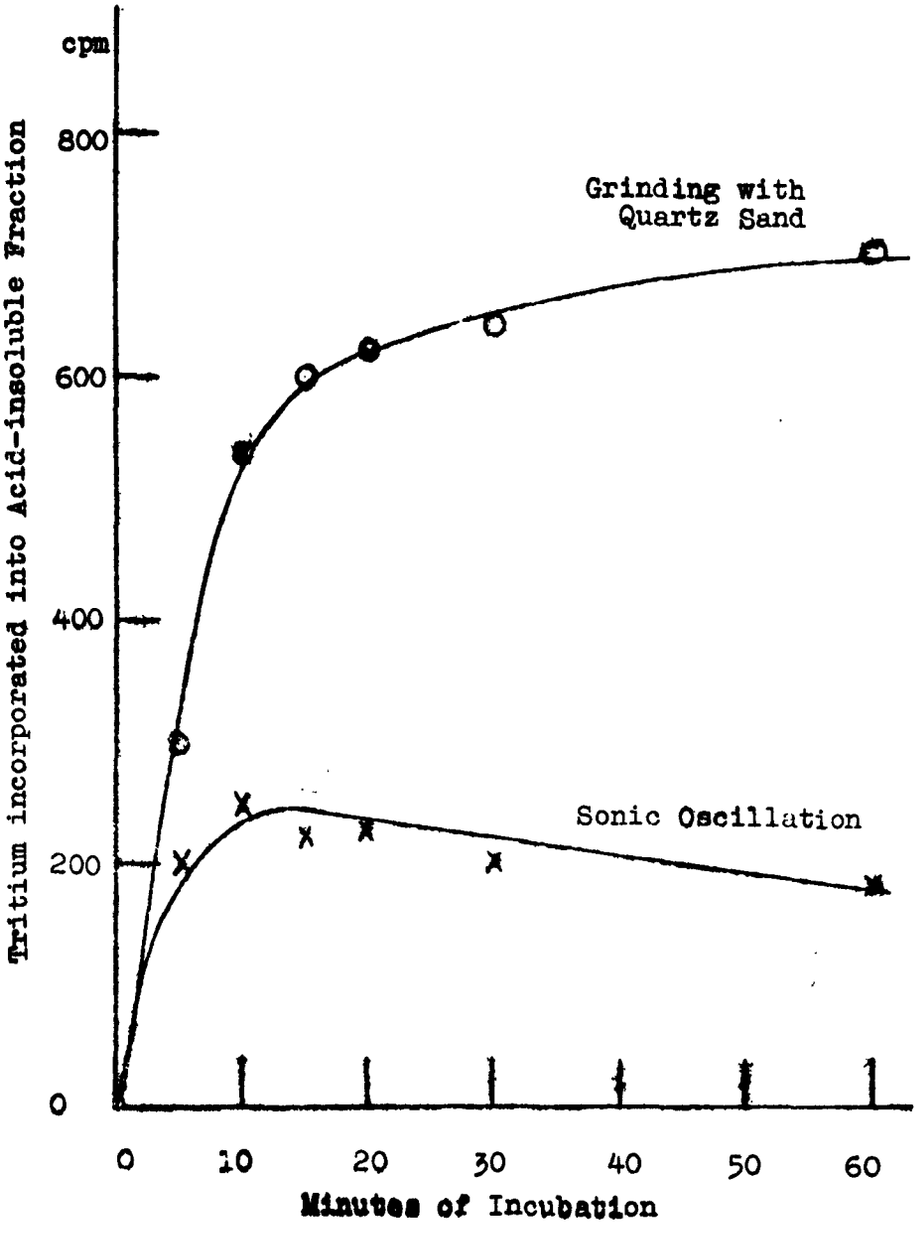
DNA Formation in the Presence of Primer DNA with Fractions obtained by Differential Centrifugation



APPENDIX "E"

Fig. 3

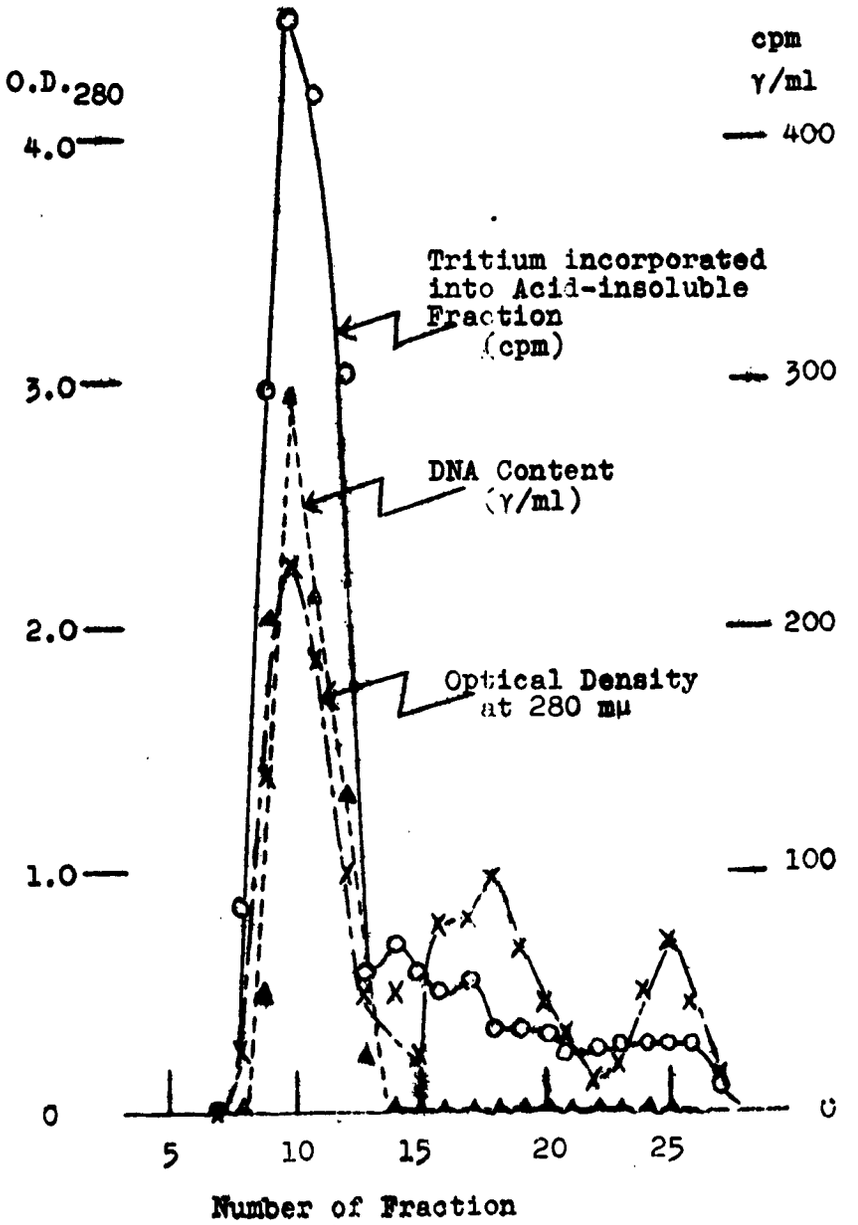
DNA Polymerizing Activity in Pellet Fraction obtained from Cell Extract prepared by Different Procedures



APPENDIX "F"

Fig. 4

Elution Diagram from a Column of Sephadex G-200 of Supernatant Fraction



APPENDIX "G"

TABLE III

The Number of Recombinants with Various Characters

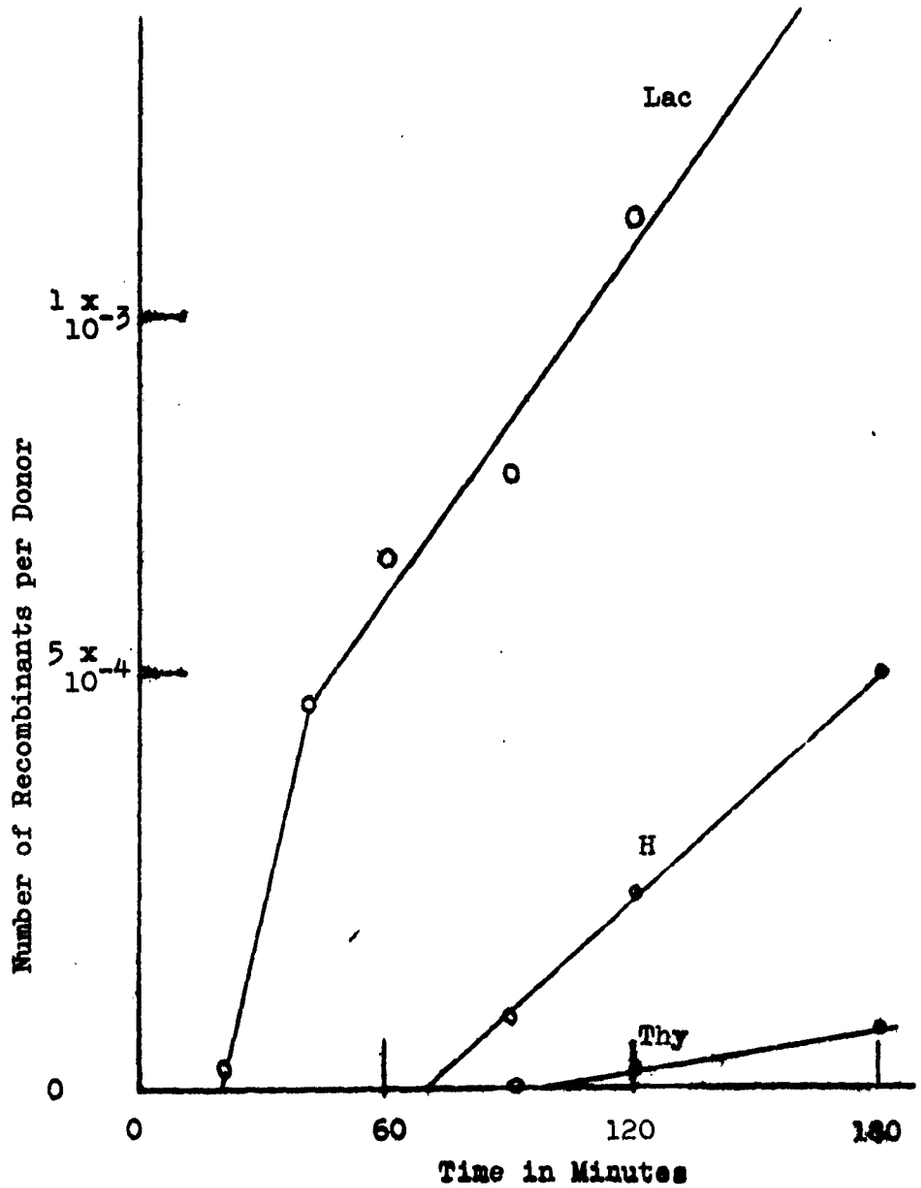
Characters				Number of Recombinants	Number* of Crossing Over
Thymine	Streptomycin	Maltose	Mannitol		
+	sensitive	+	+	12	1
+	"	+	-	11	1
+	"	-	+	2	3
+	"	-	-	16	1
+	resistant	+	+	0	3
+	"	+	-	3	3
+	"	-	+	3	3
+	"	-	-	51	1
-	sensitive	+	+	1	3
-	"	+	-	2	3
-	"	-	+	0	5
-	"	-	-	7	3
-	resistant	+	+	0	3
-	"	+	-	0	3
-	"	-	+	1	5
-	"	-	-	157	1

* Minimum number of crossing over when thymine marker locates between Sm and H markers

APPENDIX "H"

Fig. 5

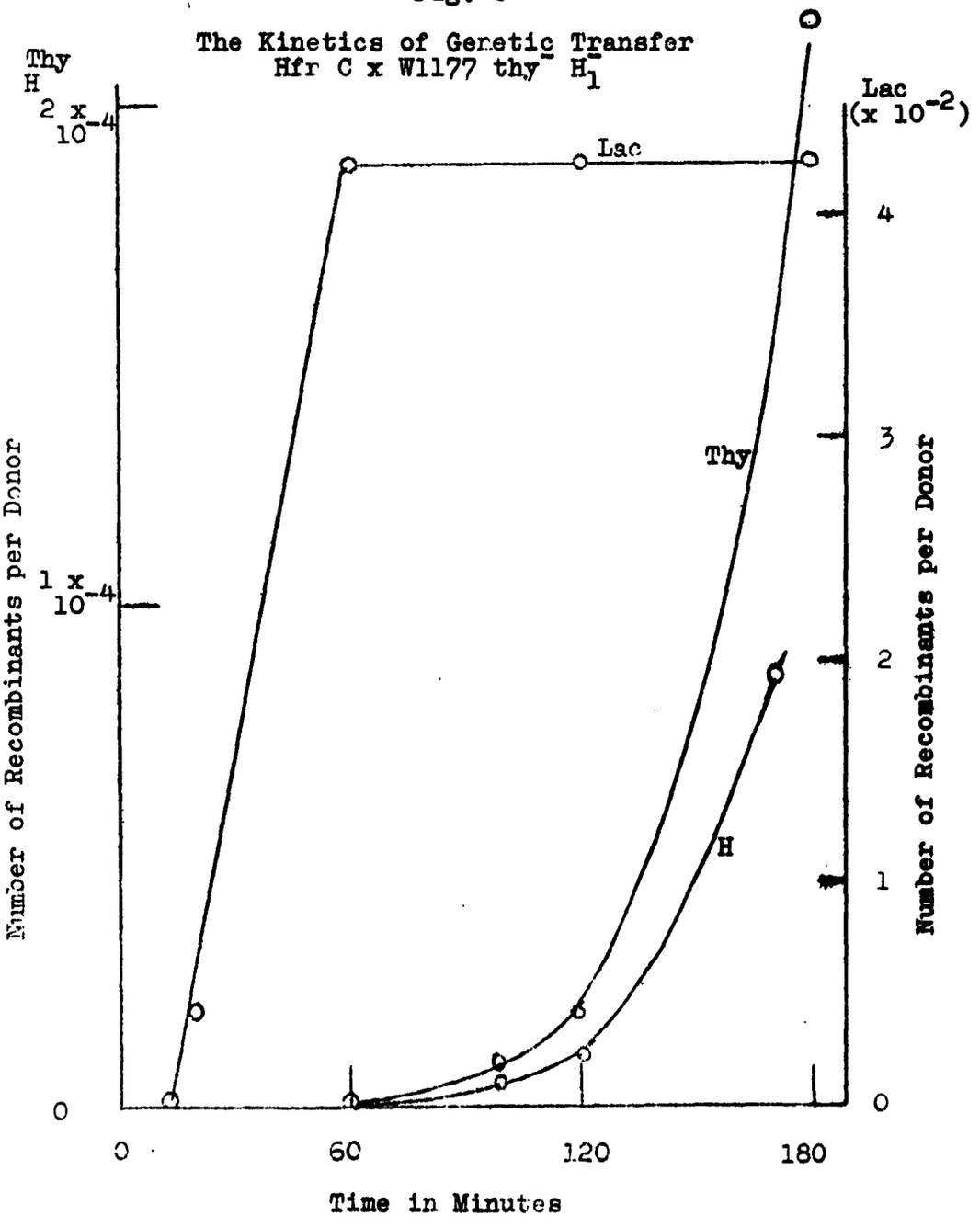
The Kinetics of Genetic Transfer
Hfr H x W 1177 thy$^-$ H$_1^-$



APPENDIX "I"

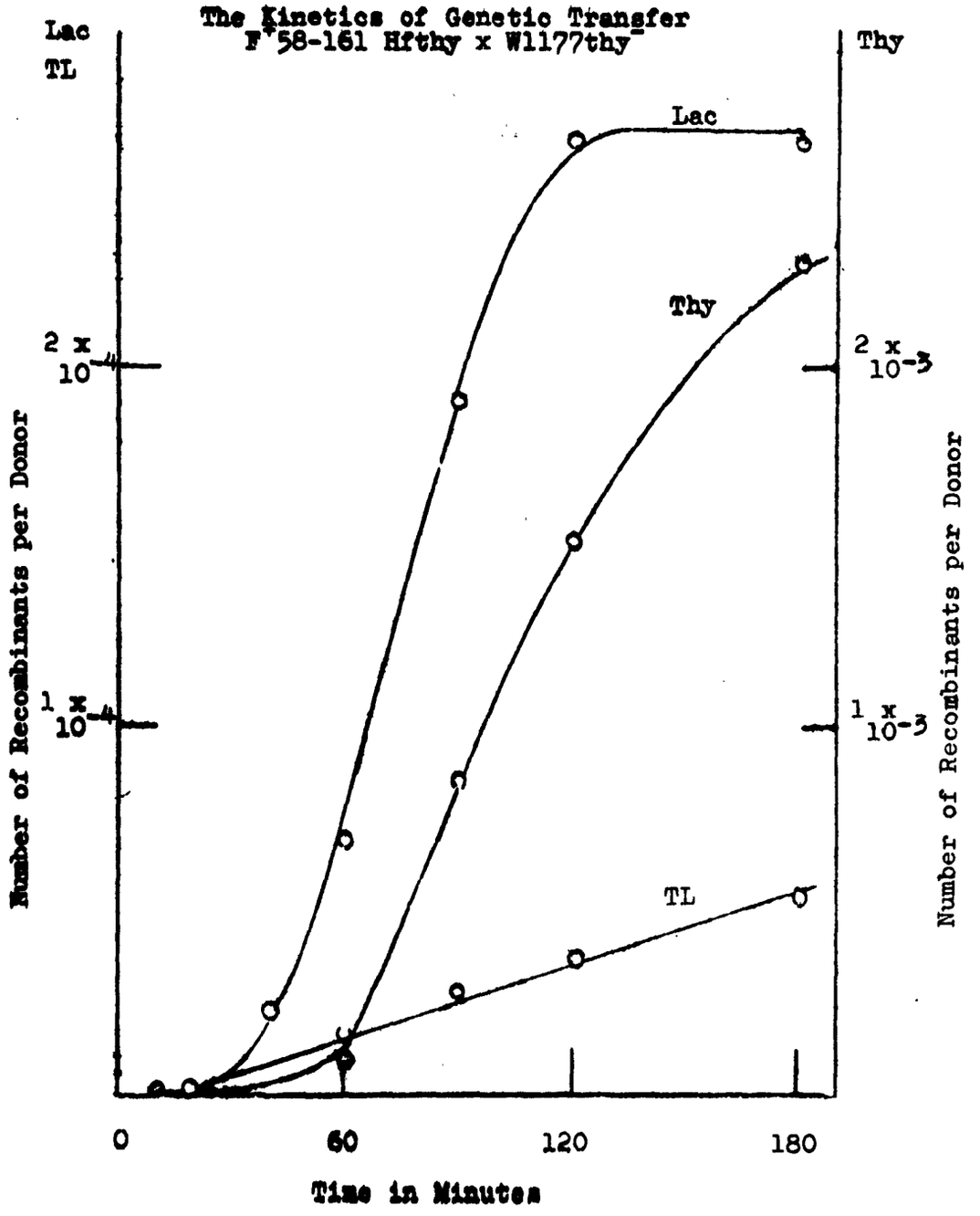
Fig. 6

The Kinetics of Genetic Transfer
Hfr C x W1177 thy⁻ H₁⁻



APPENDIX "J"

Fig. 7



APPENDIX "K"

Fig. 8

The Kinetics of Genetic Tranfser
F^+58-161 Lfthy x W1177thy$^-$